JOURNAL OF CLINICAL MICROBIOLOGY, May 2005, p. 2303–2306 Vol. 43, No. 5
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.5.2303–2306.2005

Molecular Characterization of Vancomycin-Resistant *Enterococcus faecium* Isolates from Korea

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Received 3 August 2004/Returned for modification 8 November 2004/Accepted 22 December 2004

A total of 98 vancomycin-resistant *Enterococcus faecium* (VREF) isolates from four tertiary-care hospitals in Korea during the period between 1998 and 2004 were analyzed for genotypic characteristics using the multiplex PCR, multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and *esp* gene analysis. Ninety-two isolates of VREF with VanA phenotype and five of six isolates with VanB phenotype possessed the *vanA* gene. MLST analysis revealed 9 sequence types (STs), which belonged to a single clonal complex (CC78, clonal lineage C1). Five strains showing incongruence between phenotype and genotype (VanB-*vanA*) did not belong to the same genotypic clone. The *esp* gene was detected in all VREF strains, showing 12 different *esp* repeat profiles. Data suggest that an epidemic clonal group of VREF, CC78 with *esp* gene, is also present in Asia and has differentiated into multiple diverse genotypic clones during the evolutionary process.

Since the first isolation of vancomycin-resistant *Enterococcus faecalis* and *Enterococcus faecium* in 1988 (17), vancomycin-resistant enterococci (VRE) have become one of the major threats to public health in many parts of the world. Although *E. faecalis* is more common in human infections, vancomycin resistance is more frequently observed in *E. faecium* isolates. Most vancomycin-resistant *E. faecium* (VREF) strains isolated in Korea showed the VanA phenotype, which is defined as having high-level resistance to vancomycin and teicoplanin (2). VREF isolates with the VanB phenotype characterized by variable levels of resistance to vancomycin but by susceptibility to teicoplanin have been reported in Korea since 1997 (12). Generally, the *vanA* gene cluster confers the VanA phenotype and *vanB* gene cluster is associated with the VanB phenotype. Recently, however, VRE strains with the *vanA* gene and VanB phenotype have been found in Japan, Taiwan, and Korea (3, 6, 8, 11).

VREF is an important concern not only because VREF infection is difficult to treat in clinical practice but also because VREF clones can spread within hospitals as well as between regions or countries. Molecular epidemiologic studies could clarify the genetic relatedness and molecular evolution of VREF clones. In the current study, we have investigated the phenotypic and genotypic characteristics of VREF isolates from four tertiary-care hospitals in Korea by using the broth microdilution test, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and analysis of the *esp* repeat profile.

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MATERIALS AND METHODS

VREF isolates. A total of 98 vancomycin-resistant *E. faecium* (VREF) isolates from four tertiary-care hospitals in Korea (Samsung Medical Center, Severans Hospital, Kyunghee University Hospital, and Kyungbuk National University Hospital) were analyzed in this study. The most common specimen source was urine (25 isolates) followed by rectal swab (14 isolates), blood (13 isolates), pus (9 isolates), wound (5 isolates), fluid (5 isolates), sputum (2 isolates), and tissue (2 isolates). Duplicate strains from the same patient were not included in the study. Antimicrobial resistance to vancomycin and teicoplanin were determined by measuring the MIC using broth dilution method according to the National Committee for Clinical Laboratory Standards (NCCLS) procedures (14). Genomic DNA for molecular analysis was extracted by a simple boiling-lysis method. The DNA from each of the VREF strains was analyzed by multiplex PCR method for the presence of vancomycin resistance gene (1).

MLST analysis. MLST was performed as described previously (7, 19), based on seven housekeeping genes (*atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS*, and *adk*). The allele number for each gene was assigned based on the *E. faecium* MLST database (http://efecium.mlst.net). Allelic profiles were represented as a series of 7 integers corresponding to the alleles at each of the loci, in the order of *atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS*, and *adk*. Sequence type (ST) was designated for each unique allelic profile.

***esp* gene analysis.** The presence of the *esp* gene was determined by use of a primer set (forward, 5′-GGT CAC AAA GCC CAA CTT GT-3′; reverse, 5′-ACG TCG AAA GTT CGA TTT CC-3′), which is expected to amplify 407-bp fragments (20). To determine repeat number variations of *esp* A and C repeats, two different primer combinations were used; esp_{fs}7F-esp_{fm}5R and esp_{fm}5F-esp_{fs}3R, respectively (9). The PCR products were subjected to agarose gel electrophoresis (1%) and the numbers of repeats were deduced from the sizes of the amplified fragments (9).

Pulsed-field gel electrophoresis. PFGE was performed as previously described (13). Agarose plugs containing genomic DNA were digested with SmaI (Gibco, BRL, Gaithersburg, Md.) according to the manufacturer's recommendations. Electrophoresis was performed with a CHEF-Mapper apparatus (Bio-Rad Laboratories, Milan, Italy), at 6 V/cm for 22 h. The PFGE patterns were interpreted using the criteria suggested by Tenover et al. (16).

RESULTS

Ninety-two of 98 VREF isolates (93.9%) showed the VanA phenotype with resistance to both vancomycin and teicoplanin, while six strains were resistant to vancomycin but susceptible to

TABLE 1. Genotypic characteristics of vancomycin-resistant *E. faecium* isolates from four Korean hospitals based on multilocus sequence typing and *esp* repeat profiles

ST (allelic profile)	No. of strains (%)	*esp* gene repeat profile		No. of strains (%)
		esp-A	*esp*-C	
78 (15-1-1-1-1-1-1)	58 (59.2)	7	6	1 (1.0)
		6	5	41 (41.8)
		6	4	1 (1.0)
		6	3	1 (1.0)
		5	6	8 (8.2)
		5	3	1 (1.0)
		4	5	2 (2.0)
		4	3	1 (1.0)
192 (15-1-1-1-1-7-1)	13 (13.3)	6	5	10 (12.8)
		5	7	1 (1.0)
		5	6	1 (1.0)
		4	5	1 (1.0)
203 (15-1-1-1-1-20-1)	12 (12.2)	6	5	4 (4.1)
		6	3	1 (1.0)
		5	7	2 (2.0)
		5	6	4 (4.1)
		4	3	1 (1.0)
17 (1-1-1-1-1-1-1)	7 (7.1)	6	6	1 (1.0)
		6	3	1 (1.0)
		5	8	2 (2.0)
		5	7	1 (1.0)
		4	5	2 (2.0)
204 (15-1-6-1-1-1-1)	2 (2.0)	7	6	1 (1.0)
		6	3	1 (1.0)
206 (1-3-1-1-1-1-7)	2 (2.0)	6	5	1 (1.0)
		3	4	1 (1.0)
117 (9-1-1-1-1-1-1)	1 (1.0)	5	6	1 (1.0)
205 (3-1-1-1-1-1-1)	1 (1.0)	6	3	1 (1.0)
207 (3-1-1-1-1-20-1)	1 (1.0)	6	3	1 (1.0)

teicoplanin (VanB phenotype). Of these six VREF isolates with the VanB phenotype, only one possessed the *vanB* gene, whereas five isolates showed incongruence between phenotype and genotype (VanB phenotype and *vanA* genotype).

Ninety-eight VREF isolates showed nine sequence types (STs) in MLST analysis, including five newly identified STs (STs 203, 204, 205, 206, and 207) (Table 1). The predominant ST was ST78 (58 strains, 59.2%), followed by ST192 (13.3%), ST203 (12.2%), and ST17 (7.1%). The other STs included one or two VREF isolates. STs 192, 203, 17, 204, 117, and 205 are single-locus variants of ST78. ST207 is a single-locus variant of ST203, and ST206 is a double-locus variant of ST17. Thus, the MLST data suggested that all VREF isolates in this study belong to one clonal complex, CC78 (Fig. 1).

In CC78, eight types of allelic variations have been identified due to either point mutation or recombinational process (4, 5). Three variations in the allelic profile of the *atpA* locus were due to the recombination (Fig. 1). All strains had the type 1 allele of the *purK* gene, which have been reported to be related to epidemic VREF strains (10, 18).

The *esp* gene was detected in all VREF isolates analyzed in this study. Among 98 VREF strains, the numbers of the A and C repeats of the *esp* gene varied from 3 to 7 and from 3 to 8, respectively (Table 1). Based on the *esp* A and C repeat profile, VREF isolates belonged to 12 different groups. The most prevalent *esp* profile was A6-C5 (59 isolates [60.2%]), followed by A5-C6 (14 isolates [14.3%]), A6-C3 (6 isolates [6.1%]), A4-C5 (5 isolates [5.1%]), and A5-C7 (4 isolates [4.1%]). The other *esp* profiles were displayed by only one or two strains. Isolates with the same STs showed different *esp* repeat profiles. Therefore, based on MLST and *esp* repeat profiles, a total of 29 genotypic clones were identified. Fifty-eight VREF isolates with ST78 showed eight *esp* repeat profiles, while ST192 and ST203 showed four and five different *esp* repeat profiles, respectively (Table 1). Overall, the most common genotypic clone among Korean VREF isolates was ST78 with A6-C5 *esp* repeat profile (ST78-A6-C5) (41 isolates [41.8%]). Including that clone, there were nine genotypic clones that contained multiple VREF isolates; the eight others were ST192-A6-C5 (10 isolates), ST78-A5-C6 (8 isolates), ST203-A5-C6 (4 isolates), ST203-A6-C5 (4 isolates), ST78-A4-C5 (2 isolates), ST203-A5-C7 (2 isolates), ST17-A5-C8 (2 isolates), and ST17-A4-C5 (2 isolates). Five VREF strains showing incongruence between phenotype and genotype of vancomycin resistance belonged to ST78 (4 isolates) and ST203 (1 isolate). The *esp* repeat profiles of these strains showed four different types, as follows: A4-C3 (2 isolates), A6-C5 (1 isolate), A4-C5 (1 isolate), and A7-C6 (1 isolate). Based on MLST and *esp* gene analysis, these five strains showed genetic backgrounds that were different from each other (Table 2). PFGE patterns also showed that these five strains do not belong to the same clone (Fig. 2).

DISCUSSION

In the current study, we investigated the genetic characteristics of VREF isolates from Korea by MLST, PFGE, and *esp* repeat profiles. Each method had a different ability to analyze the genotypes of VREF isolates. MLST based on seven housekeeping genes such as *atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS*, and *adk* has identified nine different STs of VREF isolates in this study, while PFGE has shown more than 32 distinguishable fragment patterns (data not shown). Isolates with different PFGE pattern belonged to the same ST on MLST. Therefore, MLST is usually useful for long-term and evolutionary process of resis-

TABLE 2. Characteristics of five vancomycin-resistant *E. faecium* isolates with VanB phenotype-*vanA* genotype

Isolate no.	Yr of isolation	Source	MIC (mg/liter)		ST (allelic profile)	*esp* repeat profile	
			Vancomycin	Teicoplanin		*esp*-A	*esp*-C
98-2	1998	Pus	>64	8	ST78 (15-1-1-1-1-1-1)	7	6
99-3	1999	Sputum	>64	8	ST78 (15-1-1-1-1-1-1)	4	3
00-1	2000	Other	>64	16	ST203 (15-1-1-1-1-20-1)	4	3
02-4	2002	Urine	64	8	ST78 (15-1-1-1-1-1-1)	6	5
03-22	2003	Rectal swab	64	4	ST78 (15-1-1-1-1-1-1)	4	5

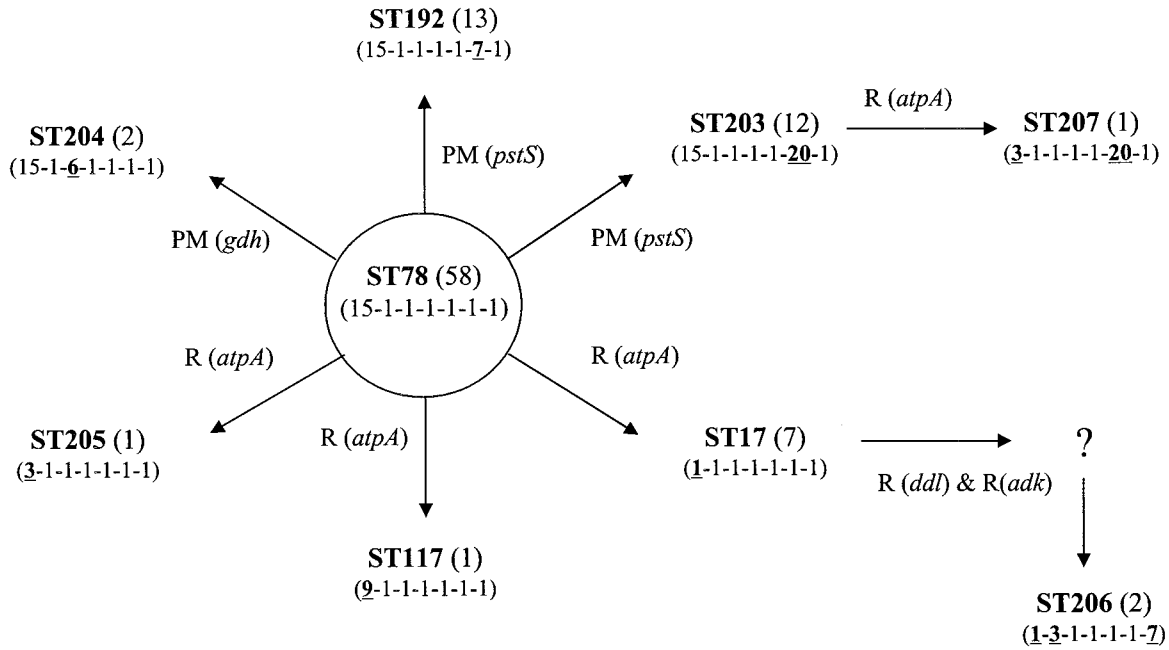

FIG. 1. Speculative evolution of vancomycin-resistant *E. faecium* isolates analyzed in this study. Alterations in the allelic profile compared with ST78 are underlined. The number of strains for each ST is indicated in parentheses. The potential reason for allelic variations is shown as R (recombination) or PM (point mutation) in the corresponding housekeeping gene (parentheses).

tant clones, and PFGE is more adequate for short-term epidemiologic study. We have also used *esp* repeat profiles to analyze the VREF isolates. Previous report showed that strains originating from a single outbreak had identical *esp* repeat profiles, which were relatively stable (9). The *esp* repeat profiles could be utilized to investigate the outbreaks of resistant clones in combination with other genotyping methods (9). In our study, combination of the *esp* repeat profiles and MLST has increased the diversity of genotypes of VREF isolates. For instance, ST78 from MLST showed eight different *esp* repeat profiles. Thus, combination of MLST and *esp* repeat profiles could be more discriminatory in evaluating the genotypes of clinical isolates of VREF.

In this study, MLST and the *esp* repeat profiles suggested the epidemic nature of VREF isolates based on STs, *purK*-1 allele, and presence of *esp* gene (7, 10). All VREF isolates in this study belonged to CC78 with the *purK*-1 allele. Previous study showed that CC78 belongs to a subgroup of *E. faecium*, lineage C1 (7). The lineage C1 represents a cluster of epidemic *E. faecium* strains that has disseminated worldwide (7, 10). The *esp* gene that encodes the virulence-associated surface protein is uniquely present in the strains of this lineage (7, 10, 18). Therefore, the presence of the *esp* gene is suggestive of epidemicity of VREF isolates (9, 19).

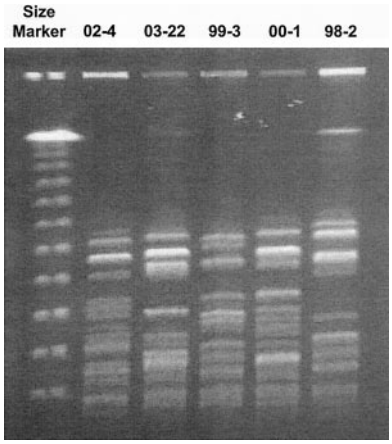

FIG. 2. SmaI macrorestriction patterns resolved in PFGE of five VREF isolates with the VanB phenotype and *vanA* genotype.

In this study, the *esp* gene was detected in all VREF strains and the *vanA* gene complex was found in 97 of 98 isolates, which suggested the clonal nature of the isolates. Therefore, our data documented that epidemic clonal group CC78 with the *esp* gene is also present in Asia as it is in Europe, the United States, and Australia (7). However, PFGE and the *esp* gene repeat profiles showed multiple genotypes of VREF isolates, which were not consistent with the result of MLST. It is assumed that one clone of ancestral VREF lineages with *esp* gene has been differentiated into multiple diverse genotypic clones during evolutionary process.

Another interesting finding from this study was incongruence between phenotype and genotype for glycopeptide resistance in five VREF isolates. Of these, one isolate has caused acute bacterial meningitis, resulting in death of patient, and other isolates were isolated from wound and urinary tract infections. These five isolates were not related either epidemiologically or genetically. Therefore, these VREF isolates with the VanB phenotype and *vanA* genotype might have occurred independently. Previous studies have suggested that point mutations in the sensor domain of *vanS* gene (3, 6, 8) or impairment of accessory proteins VanY and VanZ (3, 6, 11, 15)

would be the reason for loss of teicoplanin resistance in these strains. In the present study, three strains with the VanB phenotype and *vanA* genotype showed an IS*1216V* insertion into the *vanX-vanY* intergenic region accompanied by partial or whole deletion of *vanY* or *vanZ*. However, we could not find distinct mutations that may be responsible for teicoplanin susceptibility in another isolates. Further investigation to elucidate other molecular mechanism of incongruence is under way.

In summary, we characterized 98 VREF strains isolated from four tertiary-care hospitals in Korea by molecular typing methods. Most of the isolates showed the VanA phenotype-*vanA* genotype, but five isolates showed VanB phenotype-*van*A genotype. All VREF strains belonged to one clonal complex (CC78) which is related to a globally epidemic *E. faecium* clone. Based on MLST and the *esp* gene repeat profiles, this clone might have been differentiated into 29 genotypic clones. The combination of MLST and the *esp* repeat profiles would be useful for genetic characterization of VREF isolates with regard to the evolutionary process and epidemicity of the clones.

ACKNOWLEDGMENT

This study was supported by the Asian-Pacific Research Foundation for Infectious Diseases.

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